

Detection of SPM and IMP metallo- β -lactamases in clinical specimens of *Pseudomonas aeruginosa* from a Brazilian public tertiary hospital

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ABSTRACT

Phenotypic and genotypic SPM and IMP metallo- β -lactamases (MBL) detection and also the determination of minimal inhibitory concentrations (MIC) to imipenem, meropenem and ceftazidime were evaluated in 47 multidrug-resistant *Pseudomonas aeruginosa* isolates from clinical specimens. Polymerase chain reaction detected 14 positive samples to either *bla*_{SPM} or *bla*_{IMP} genes, while the best phenotypic assay (ceftazidime substrate and mercaptopropionic acid inhibitor) detected 13 of these samples. Imipenem, meropenem and ceftazidime MICs were higher for MBL positive compared to MBL negative isolates. We describe here the SPM and IMP MBL findings in clinical specimens of *P. aeruginosa* from the University Hospital of Botucatu Medical School, São Paulo, Brazil, that reinforce local studies showing the high spreading of *bla*_{SPM} and *bla*_{IMP} genes among Brazilian clinical isolates.

Keywords: *Pseudomonas aeruginosa*; drug resistance, bacterial; carbapenems; metalloproteins; polymerase chain reaction.

INTRODUCTION

The most common cause of Gram-negative bacterial resistance to β -lactam antimicrobial agents is the β -lactamases production, which is highly diversified and spread in several bacteria.^{1,2} Metallo- β -lactamase (MBL) belongs to Group 3 of enzymes proposed by Bush and Jacoby,² and shows ability to hydrolyze carbapenems instead of cephalosporins, cephamycins, and penicillins. Contrasting to serine- β -lactamases, MBL has poor ability to hydrolyze monobactams.² *Pseudomonas aeruginosa* is one of the most common causes of nosocomial infections, persisting at hospital environment and acquiring mobile elements of resistance.³ The emergency of MBL in clinical isolates of *P. aeruginosa* is worrisome, due to the reduction of therapeutic options and the spreading ability of this bacterium. In addition, longer length of hospital stay and high mortality rates are associated to MBL producing *P. aeruginosa* infections.³ Besides β -lactam resistance, *P. aeruginosa* susceptible only to colistin or polymyxin B has been described³⁻⁵ in several infectious processes, which demand the increase use of these drugs. An accurate, easy-to-perform and fast phenotypic test to detect MBL is

important to implement infection control measures, and to apply the most appropriate therapy.^{6,7} Several studies analyzed different substrata and MBL inhibitors associations researching MBL-producing bacteria,^{7,8} but no standardization to MBL detection has been published so far by guidelines, namely Clinical and Laboratory Standards Institute (CLSI). E-test strips have also been employed to detect MBL enzymes.⁸ In Brazil, São Paulo metallo- β -lactamase (SPM) and imipenemase (IMP) are prevalent in *P. aeruginosa*.^{7,9,10} In the present study, we aimed to detect MBL production in clinical specimens of *P. aeruginosa* isolated from patients of a Brazilian public tertiary hospital, and providing data to epidemiology of MBL in Brazil.

Forty-seven *P. aeruginosa* strains isolated between 2006 and 2007 were evaluated. The isolates were recovered from clinical specimens collected from patients attended at University Hospital of Botucatu Medical School, São Paulo State, Brazil, a regional reference tertiary hospital. Isolates were chosen due to their multidrug-resistant profile, according to disk-diffusion susceptibility test and CLSI cutoffs,¹¹ and the sole susceptibility to polymyxin B, confirmed by E-test (AB Biodisk) strips [minimal inhibitory

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concentrations (MIC) ranging from 0.094 to 2; MIC₅₀: 0.5; MIC₉₀: 1.5 µg/mL; 100% susceptible]. MICs were determined (in duplicate) by microdilution broth method for imipenem (ABL), meropenem (Astra Zeneca) and ceftazidime (Nova-Farma); quality controls were performed with *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 strains. MBL phenotypic detection was evaluated by the double-disk synergy test (DDST) with imipenem or ceftazidime disks (Oxoid) as substrata, and mercaptopropionic acid (Sigma-Aldrich), mercaptoacetic acid (Sigma-Aldrich), or ethylenediamine tetra-acetic acid (EDTA, Sigma-Aldrich) as MBL inhibitors, according to Picão et al.⁷ Genotypic detection targeting to the *bla*_{SPM} and *bla*_{IMP} genes was carried out by polymerase chain reaction (PCR), according to protocols published elsewhere.⁹ Positive and negative quality control strains were included to assess the quality of phenotypic and genotypic assays. Student's *t* test was carried out to compare the median MIC of positive and negative MBL strains; sensitivity and specificity were calculated for the different substrata antimicrobials and MBL inhibitors assessed in the DDST; *p*-values below 0.05 were considered statistically significant. The study was approved by the local Ethics Committee (Protocol 2886-2008).

Forty-seven isolates of *P. aeruginosa* were analyzed, from urine (17), blood (5), and various other specimens (25). The distribution of isolates across the hospital was diversified, being, even in low numbers, mostly isolated from Emergency Unit and Urology Wards (5 each), and Central Intensive Care Unit (4). MIC values range (and MIC that inhibited the growth of 90% of the strains, MIC₉₀) for imipenem, meropenem and ceftazidime, were, respectively, 16 to 512 (512); 16 to > 512 (512); 4 to > 512 (512 µg/mL). MIC median for MBL-positive strains were significantly higher than those MIC for MBL-negative strains (*p* < 0.0001), for each drug: imipenem, meropenem and ceftazidime (Figure 1). Polymyxin B MIC median also showed difference between MBL-positive and -negative strains (0.98 vs. 0.66 µg/mL, respectively; *p* = 0.0097). MBL genotypic detection was carried out with specific primers for *bla*_{SPM} and *bla*_{IMP} genes. Fourteen (29.8%) isolates in 47 samples were either *bla*_{SPM} or *bla*_{IMP} positive. *bla*_{SPM} gene accounted for 71% (10/14) positive MBL strains, while *bla*_{IMP} was detected in 29% (4/14). DDST employing mercaptopropionic acid and ceftazidime showed the best association to PCR results, with sensitivity of 92.8% and specificity of 100%. MBL-positive *P. aeruginosa* strains were isolated from different clinical sources (urine, blood, and other biological fluids) and from several wards: Gastric Surgery, Gastroenterology, Intensive Care Unit, Intern Medicine, Orthopedics, Urology and Agreement Ward.

P. aeruginosa is an important nosocomial agent, and several resistance mechanisms have been related on this microorganism;^{12,13} MBL has been emerging as a worrying resistance mechanism in this and in several other Gram-

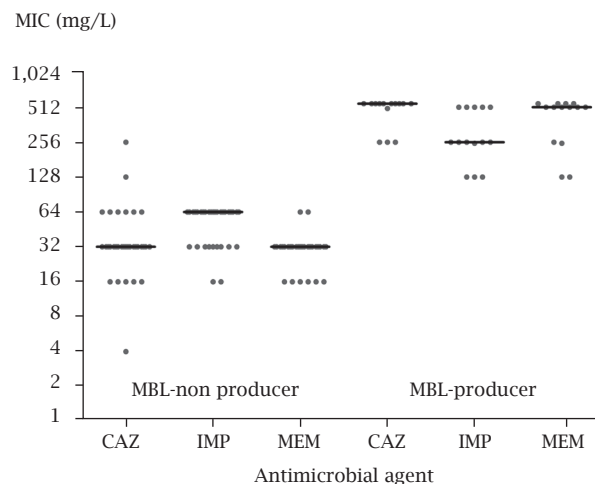


Figure 1: Comparison between MIC of MBL-negative and positive- *P. aeruginosa* strains. o represents each isolate; horizontal bars represent average values. CAZ, ceftazidime; IMP, imipenem; MEM, meropenem.

negative rods.² β -lactamases are currently classified by two ways: based on the amino acid sequence (molecular classification) or based on the hydrolytic and inhibition properties (functional classification).² According to molecular classification β -lactamases are grouped into class A, B, C and D. While class A, C and D enzymes are characterized to utilize serine for β -lactam hydrolysis, class B enzymes are metalloenzymes, that use divalent cations as cofactors to substrate hydrolysis.² Functional classification divides β -lactamases enzymes into 3 groups: group 1, cephalosporinases; group 2, the largest one, which contains enzymes that hydrolyses penicillins, cephalosporins, monobactams, carbenicillin, cloxacillin, and carbapenems; and group 3, metallo-carbapenemase. Colistin-only susceptible *P. aeruginosa* is a relatively contemporary issue, once carbapenems remained as effective therapeutic choice until the carbapenemases emergence on the last two decades.^{7,8,14} Investigating MBL-encoding genes, we found isolates carrying *bla*_{SPM} and *bla*_{IMP} genes that together correspond to 29.8% of evaluated samples. In Brazil, the dissemination of *P. aeruginosa* carrying *bla*_{SPM} gene has been reported throughout from several regions,^{15,16} and SPM is the prevalent MBL among the isolates, although other enzymes have also been described, but with low frequency.^{9,10,16,17} SPM carbapenemase was the main mechanism that conferred resistance to ceftazidime on ceftazidime-resistant *P. aeruginosa* isolated from bloodstream infections.¹⁰ MBL rate found here is higher than that one reported by Viana Vieira et al.¹⁴ (7.5%), but it is lower than those published by other Brazilian researchers (35.9 to 80.4%).^{9-11,18-20} Zavaski et al.³ found 30 *P. aeruginosa* only susceptible to polymyxin B among 86 MBL producing strains and the *bla*_{SPM} gene was detected in all the 14 strains randomly selected, and

Gräf et al.¹⁹ also detected the *bla*_{SPM} gene in strains only susceptible to polymyxin B, both from Brazilian hospitals. Our data are in accordance to these studies, showing that SPM is the prevalent MBL among Brazilian *P. aeruginosa* isolates. The lower frequency of MBL in our patient population might be the result of our inclusion criteria (only susceptibility to polymyxin B), once that resistance to imipenem and meropenem simultaneously suggests the evolvement of carbapenem resistance mechanism other than the enzymatic one, such as porin loss and/or overexpression of efflux pumps.¹⁰ High MIC values for carbapenems and ceftazidime are associated to MBL production, and this may be a useful tool to differentiate from other resistance mechanism, such as efflux pumps or chromosomal inducible AmpC β -lactamase.¹ Recent studies confirm this tendency,^{5,9,10,12} and our data also showed this remark (Figure 1). Some peculiar serine- β -lactamases, such as GES-5, can also present hydrolytic activity against carbapenems in *P. aeruginosa* isolates; this activity is, however, lower than that one presented by MBL-encoding genes isolates, as recently reported in a Brazilian study.¹⁰ Resistance to other antimicrobial agent classes^{12,13} reduces the therapeutic options to manage multidrug-resistant *P. aeruginosa* clinical isolates, and it seems to be even more recurrent to employ last resource drugs, such as polymyxins, to treat these infections. Polymyxin B showed efficacy against all of the isolates here evaluated. Similar observation was reported by Lee et al.⁵ who studied 17 colistin-only sensitive isolates from Korea (MIC ranging from 0.5-2.0 μ g/mL). In imipenem-resistant *P. aeruginosa* strains isolated from a Brazilian tertiary care university hospital, polymyxin B showed great activity (100% susceptible; MIC₉₀ \leq 0.5 mg/L).⁹ However, we can wonder how much longer these bacteria will persist susceptible to these remaining drugs if this selective pressure goes on. Comparing different phenotypic tests to detect MBL, we found that ceftazidime disk and mercaptopropionic acid association, in a double disk synergy test, was the most sensitive and specific assay, as it was reported by Picão et al.⁷ We observed that all other combinations of substrata and inhibitors (imipenem and mercaptopropionic acid; imipenem and mercaptoacetic acid; imipenem and EDTA; ceftazidime and mercaptoacetic acid; ceftazidime and EDTA) showed maximum specificities (100%), but low sensitivity to these combinations (zero to 71.4%), restricting their utilization. In spite of the high cost, MBL E-test strips can be employed to detect the MBL-producing *P. aeruginosa* due its high sensibility and specificity.⁸ Despite the recommendation for screening and confirming carbapenemase production in Enterobacteriaceae, CLSI document M100-S20¹¹ made no mention of detecting this enzyme on non-fermentative Gram-negative bacteria. Early identification of MBL in clinical specimens is important to provide correct antimicrobial therapy guidance, once appropriate therapy implementation can nullify the worst prognostic of MBL producing

P. aeruginosa.³ When detected, it is possible to avoid the dissemination of such strains among patients⁷⁻⁹ and to avoid the MBL genetic determinants spread to different species of Gram-negative bacteria, as Enterobacteriaceae.⁶ The contention of MBL producing *P. aeruginosa* is highly recommended, because patient-to-patient transmission may lead these strains to be endemic in such institution³ or among different hospitals.¹⁵⁻¹⁷ In Brazil, Gales et al.¹⁶ reported the spreading of *bla*_{SPM}-producing *P. aeruginosa* clone throughout the country. The non-centralized distribution of positive MBL *P. aeruginosa* from Hospital of Botucatu Medical School (i.e., the recovering of MBL *P. aeruginosa* from hospitalized patients from several wards) suggests the non-clonal spreading of *P. aeruginosa* strains, although further molecular analyses are necessary to confirm this hypothesis. Our results contribute to the study of MBL epidemiology in Brazil, mainly to *bla*_{SPM} spreading. It is worthwhile to emphasize that, after restricted by more than ten years to Brazilian hospitals, SPM seems to become a global challenge,^{15,20} warning for the role of human traffic in spreading MBL genes.^{6,20}

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